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ORIGINAL ARTICLE

Formation of syringyl-rich lignins in maize as influenced by feruloylated xylans and *p*-coumaroylated monolignols

John H. Grabber · Fachuang Lu

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Abstract Grass cell walls are atypical because their xylans are acylated with ferulate and lignins are acylated with *p*-coumarate. To probe the role and interactions of these *p*-hydroxycinnamates during lignification, feruloylated primary cell walls isolated from maize cell suspensions were lignified with coniferyl and sinapyl alcohols and with varying levels of *p*-coumarate esters. Ferulate xylan esters enhanced the formation of wall-bound syringyl lignin more than methyl *p*-coumarate, however, maximal concentrations of syringyl lignin were only one-third that of guaiacyl lignin. Including sinapyl *p*-coumarate, the presumed precursor of *p*-coumaroylated lignins, with monolignols unexpectedly accelerated peroxidase inactivation, interfered with ferulate copolymerization into lignin, and had minimal or adverse effects on cell wall lignification. Free phenolic groups of *p*-coumarate esters in isolated maize lignin and pith cell walls did not undergo oxidative coupling with each other or with added monolignols. Thus, the extensive formation of syringyl-rich lignins and the functional role of extensive lignin acylation by *p*-coumarate in grasses remains a mystery.

Keywords Cell wall · *p*-Coumarate · Cross-linking · Ferulate · Lignin · Peroxidase

J. H. Grabber (✉) · F. Lu
U.S. Dairy Forage Research Center,
Agricultural Research Service, U.S. Department of Agriculture,
1925 Linden Drive West, Madison, WI 53706, USA
e-mail: jgrabber@wisc.edu

F. Lu
Department of Agricultural and Biological
Systems Engineering, University of Wisconsin,
Madison, WI 53706, USA

Abbreviations
AIP 2-Aminoindan-2-phosphonic acid

Introduction

Grasses have unusually high concentrations of ferulic and *p*-coumaric acids ester-linked to cell wall polymers. Depending on the tissue and its stage of development, C4 grasses tend to have greater concentrations of these *p*-hydroxycinnamic acids than C3 grasses; cell walls in maize (*Zea mays* L.) and other C4 grasses contain up to 4% ferulate and up to 3% *p*-coumarate (Hatfield et al. 1999; Saulnier et al. 1999).

Xylans, acylated at the C5-OH of α-L arabinosyl moieties with ferulate, are formed intracellularly in grasses and then deposited into primary and secondary cell walls of a variety of tissues (Gordon et al. 1985; Myton and Fry 1994; Migne et al. 1998; Hatfield et al. 1999; MacAdam and Grabber 2002). Xylans are cross-linked by peroxidase-mediated coupling of ferulate monomers into a complex array of dimers and trimers and by extensive copolymerization of ferulates into lignin (Grabber et al. 2004). Ferulate and diferulate cross-links contribute to cell wall stiffening, anchoring of lignin in cell walls, cessation of plant growth, enhanced plant pest resistance, and reduced enzymatic hydrolysis of cell walls (Schopfer 1996; Bergvinson et al. 1997; Grabber et al. 1998; Casler and Jung 1999; MacAdam and Grabber 2002; Bily et al. 2003). Ferulate and diferulates may also act as nucleation sites for lignin formation (Ralph et al. 1995; Grabber et al. 2002) and they play a role in the physical and antioxidant properties of foods (Garcia-Conesa et al. 1999; Schooneveld-Bergmans et al. 1999; Ferguson et al. 2003).

Most *p*-coumarates in cell walls are esterified to the γ-position of phenylpropanoid side-chains of mainly

syringyl units in lignin (Ralph et al. 1994; Grabber et al. 1996a). Little is known about the biosynthesis of *p*-coumarate units in lignin, but structural studies suggest that syringyl units are enzymatically pre-acylated with *p*-coumarate prior to their incorporation into lignin (Lu and Ralph 2005), thus sinapyl *p*-coumarate is the logical precursor. Based on the analysis of isolated lignins and whole cell walls, *p*-coumarate can comprise up to 20% of the lignin in some C4 grass tissues (Ralph et al. 1994; Hatfield et al. 1999). *p*-Coumarate concentrations vary considerably between tissues, with extremely low levels in mesophyll and epidermis and moderate to high levels in pith parenchyma, sclerenchyma, and vascular tissues (Gordon et al. 1985; Chesson et al. 1997; Hatfield et al. 1999). *p*-Coumarate esters on lignin are thought to form few cross-linked structures mediated by radical coupling reactions and most remain as terminal units with an unsaturated side chain and a free phenolic group (Ralph et al. 1994).

The deposition of *p*-coumaroylated lignins is associated with growth cessation in grasses (Musel et al. 1997; MacAdam and Grabber 2002) and both extractable and cell wall bound *p*-coumarates are thought to play a role in pest resistance (Santiago et al. 2005, 2006). In vitro studies also suggest that catalytic amounts of apoplastic *p*-coumarate enhance the generation of hydrogen peroxide from NADH by apoplastic peroxidases and the oxidative coupling of sinapyl alcohol into syringyl lignins in maize and other plant species (Takahama et al. 1996; Hatfield et al. 1997; Sukalovic et al. 2005). However, since grass lignins are highly acylated with *p*-coumarate, the role of *p*-coumarate in lignification has probably not been fully ascertained. In this study, we examined the copolymerization of monolignols and *p*-coumarate esters in feruloylated primary maize cell walls and in isolated maize lignin to probe the function and interactions of *p*-hydroxycinnamates during the formation of syringyl and mixed syringyl–guaiacyl lignins.

Materials and methods

Lignification with varying levels of ferulate and methyl *p*-coumarate

Nonlignified maize cell suspensions (cv. Black Mexican) were grown with 0 or 5 μg/ml of 2-aminoindan-2-phosphonic acid (AIP) for 14 days (Grabber et al. 1998). Two replicates of isolated cell walls (~0.28 g dry weight) were stirred in 30 ml of PIPES buffer (75 mM; pH 6.8) with 1,500 U of glucose oxidase (Type VII-S, Sigma, EC 1.1.3.4) to generate H_2O_2 via added glucose. Cell walls were artificially lignified (Grabber et al. 1996b) over a 25 h period via wall bound peroxidase by drop-wise addition of 0.64 mmol glucose, 0.57 mmol of sinapyl alcohol, and 0 or 0.028 mmol of methyl *p*-coumarate dissolved in 50 ml of 5% aqueous ethanol. For comparison, cell walls from cell suspensions grown without AIP were lignified in the same manner with 0.64 mmol glucose and 0.57 mmol of coniferyl alcohol. Nonlignified controls were stirred in the reaction medium without the addition of glucose or lignin precursors.

Lignification with varying levels of sinapyl *p*-coumarate

Primary cell walls (~0.9 g dry weight) from 14 days old maize cell suspensions (Grabber et al. 1996b) were stirred in 70 ml of HOMOPIPES buffer (30 mM; pH 5.5) and artificially lignified over 30 or 45 h by the drop-wise addition of lignin precursors (0.6 or 0.9 mmol) and H_2O_2 (0.8 or 1.2 mmol). The lignin precursors were coniferyl or sinapyl alcohols added alone or in a 2:1 molar ratio with sinapyl *p*-coumarate. Lignin precursors were prepared as 0.013 mmol/ml solutions in 35% (v/v) dioxane–water and H_2O_2 was prepared as a 0.016 mmol/ml solution in water. The two levels of precursor addition, used to increase the scope of inference of the study, were considered as replications for each treatment (Steel and Torrie 1980). Nonlignified controls were stirred in a 3:1:4 (v/v/v) mixture of buffer:dioxane:water, similar to the final makeup of the lignification reaction media.

Lignification with varying levels of ferulate and sinapyl *p*-coumarate

Maize cell suspensions were grown for 14 days with 0, 2, or 10 μg/ml of AIP to vary cell wall feruloylation (Grabber et al. 1998). Isolated cell walls (~1.4 g dry weight) were stirred in 120 ml of water and H_2O_2 (0.25 mmol, ~2.5 Eq/mol of cell wall ferulate) in 6 ml of water was added drop-wise for 1 h to dimerize ferulates via wall bound peroxidase. Cell walls were then artificially lignified over a 12.5 or 25 h period by drop-wise addition of lignin precursors (0.7 or 1.4 mmol) and H_2O_2 (0.8 or 1.6 mmol). The lignin precursors were coniferyl alcohol, sinapyl alcohol, and sinapyl *p*-coumarate added in molar ratios of 3:4:0, 3:3:1, and 3:2:2. Lignin precursors were prepared as 0.009 mmol/ml solutions in 35% (v/v) dioxane and water and H_2O_2 was prepared as a 0.010 mmol/ml solution in water. The two levels of precursor addition, used to increase the scope of inference of the study, were considered as replications for each treatment (Steel and Torrie 1980). After adding H_2O_2 to dimerize ferulates, nonlignified controls were stirred in a 1:8 (v/v) mixture of dioxane:water, similar to the final makeup of the lignification reaction media.

p-Coumarate coupling in preformed lignins

Partially lignified pith tissues from greenhouse-grown B73 inbred maize (V16 stage) were frozen in liquid N, pulverized in a Waring blender, and then blended for 1 min with ice-cold PIPES buffer (10 mM, pH 7.0) containing 100 mM $CaCl_2$. The slurry was collected on Miracloth and washed repeatedly with PIPES buffer followed by water to remove cytoplasmic contents. Pith cell walls (~180 mg dry weight) were stirred in 50 ml of HOMOPIPES buffer (25 mM; pH 5.5) with 1,000 U of glucose oxidase (Type VII-S, Sigma) to generate H_2O_2 via added glucose. Then 0.17 mmol glucose or 0.5 mmol glucose + 0.33 mmol of coniferyl or sinapyl alcohols prepared in 50 ml of buffer were added dropwise to cell walls over a 25-h period. Control cell walls were incubated in buffer without glucose oxidase, glucose, or monolignols. Treatments were replicated thrice by using pith isolated from the 3rd and 5th elongated internodes and the 7th partially elongated internode above ground level.

Maize lignin (5 mg) isolated from rind tissue (Ralph et al. 1994) was dissolved in 100 µl of 80% dioxane–water (v/v) and mixed in screw-cap culture tubes with 3.9 ml of HOMOPIPES buffer (10 mM, pH 5.5) or 50% dioxane–HOMOPIPES buffer (v/v). Two replicates of lignin suspensions then received 6 hourly additions of the following four treatments: (1) horseradish peroxidase (5 U, Sigma, Type II in 25 µl of water), dioxane (50 µl) and water (25 µl); (2) peroxidase, dioxane, and H_2O_2 (1.5 µmol in 25 µl of water); (3) peroxidase, coniferyl alcohol (0.7 µmol in 50 µl of dioxane) and H_2O_2; or (4) peroxidase, sinapyl alcohol (0.7 µmol in 50 µl dioxane) and H_2O_2. One hour after the final addition, 4.6 ml of 4 M NaOH (containing 0.5 mg 2-OH cinnamic acid as an internal standard) was added to each tube and mixed. Solutions in each tube were bubbled with N_2 gas for 60 s and then the tubes were capped and incubated for 20 h at 25°C. After incubation, solutions were acidified to pH <2 with HCl, cooled, and then extracted thrice with 5 ml of ethyl acetate for analysis of *p*-hydroxycinnamates as described below.

General methods

Coniferyl and sinapyl alcohols, methyl *p*-coumarate, and sinapyl *p*-coumarate were prepared according to published methods (Lu and Ralph 1998b; Lu and Ralph 1998a). Other chemicals were purchased from commercial sources. During the course of lignification experiments, the activity of peroxidase was monitored visually by adding 100 µl each of 20 mM guaiacol and 20 mM glucose or H_2O_2 to 500 µl of reaction medium. After precursor additions were completed, cell walls were stirred for an additional 24–72 h before collection on glass-fiber filters (1.2 µm porosity) and washing with water followed by acetone to remove non-bound dehydrogenation products. After evaporating off acetone in a hood, cell walls were dried at 55°C and weighed. Filtrates from cell walls lignified with sinapyl *p*-coumarate were evaporated in vacuo to remove acetone, acidified to pH 3.8 with HCl, and extracted with ethyl acetate to isolate dehydrogenation products. Extracts were dried with anhydrous magnesium sulfate, filtered, and evaporated in vacuo. Alternatively, dehydrogenation products in water washes were bound to ENVI-18 columns and eluted with MeOH. Acetone washes were also evaporated in vacuo to recover dehydrogenation products. Isolated dehydrogenation products were dissolved in DMSO and analyzed by ^{1}H-NMR.

Acid-insoluble Klason lignin in cell walls was determined by a two-stage hydrolysis in 12 M H_2SO_4 at 25°C for 2 h followed by 1.6 M H_2SO_4 at 100°C for 3 h (Hatfield et al. 1994). Ester-linked *p*-hydroxycinnamate monomers and dimers released from cell walls and filtrate subsamples by 2 M NaOH (20 h, 25°C) were extracted with ethyl ether, silylated, and quantified by GLC-FID and verified by GLC-MS (Grabber et al. 2000; Bunzel et al. 2001). All analyses were carried out in duplicate. Data were subjected to analysis of variance and, unless noted otherwise, all reported differences were significant at $P = 0.05$.

Results

Lignification with varying levels of ferulate and methyl *p*-coumarate

Previous in vitro studies (Takahama et al. 1996; Hatfield et al. 1997) revealed that catalytic amounts of *p*-hydroxycinnamates such as methyl *p*-coumarate dramatically enhance oxidative coupling of sinapyl alcohol by apoplastic peroxidases in some plants including maize. Therefore, we examined whether improved oxidation of sinapyl alcohol leads to greater in situ formation of syringyl lignin in maize cell walls. In this study, nonlignified primary cell walls containing in situ peroxidases and ferulate xylan esters were isolated from maize cell suspensions and artificially lignified with sinapyl alcohol or sinapyl alcohol + catalytic amounts of methyl *p*-coumarate (1:0.05 mol/mol). In order to differentiate the effects of ferulate from methyl *p*-coumarate on syringyl lignin formation, we manipulated xylan feruloylation by growing cell suspensions with varying levels of AIP, a specific inhibitor of phenylalanine ammonia lyase (Grabber et al. 1998). Growing cell suspensions with AIP decreased cell wall concentrations of alkali-labile (ester-linked) ferulate from 11.15 to 1.72 mg/g and alkali-labile diferulates from 4.27 to 1.49 mg/g and it shifted the molar ratio of added methyl *p*-coumarate to cell wall

ferulates from 1.4 to 7 (data not shown). Alkali-labile *p*-coumarate was also a minor component of maize cell walls and AIP treatment decreased its concentration from 0.167 to 0.082 mg/g.

Although excess sinapyl alcohol was added to form up to 300 mg/g of syringyl lignin, only 86 mg/g of acid-insoluble Klason lignin was recovered in cell walls with normal xylan feruloylation. By contrast, lignification with coniferyl alcohol yielded 267 mg/g of Klason lignin, indicating a near quantitative formation of wall-bound guaiacyl lignin. Cell walls lignified with coniferyl alcohol also had a somewhat greater incorporation of ferulate and diferulates into cross-linked structures with lignin (Table1). Following precursor addition, high levels of peroxidase activity were detected in all treatments, thus precluding this as a factor affecting lignification. Klason lignin levels were reduced 30 mg/g by AIP treatment and about one-third of this decline was due to fewer ferulates being incorporated into lignin. Adding catalytic amounts of methyl *p*-coumarate with sinapyl alcohol increased Klason lignin concentrations by only 6 mg/g in cell walls with normal or reduced feruloylation. Methyl *p*-coumarate additions did not affect the quantity of ferulate and diferulates copolymerized into lignin.

Lignification with varying levels of sinapyl *p*-coumarate

In the next study, coniferyl or sinapyl alcohols were added alone and with sinapyl *p*-coumarate to assess how the likely precursor of *p*-coumarolyated lignin influences guaiacyl and syringyl lignin formation in maize. Precursors were added to primary cell walls from maize cell suspensions to potentially form lignin at onefold to 1.5-fold the concentration found in mature maize parenchyma (Morrison et al. 1998). When added, sinapyl *p*-coumarate comprised one-third of the added precursors to reflect its presumed abundance in maize lignin (Ralph et al. 1994). Since the water solubility of sinapyl *p*-coumarate (~0.015 mg/ml) was about 200-fold less than monolignols, all precursors were prepared and found to be stable in 35% (v/v) dioxane–water. Surprisingly, UV-scans revealed that sinapyl *p*-coumarate degraded within 30 min of addition to neutral buffer but it was stable for about 1–2 h in slightly acidic (pH 5.5) buffer or water (data not shown). Therefore in this and subsequent studies, we gradually added precursors prepared in dioxane–water to cell walls suspended in slightly acidic buffer or water.

Based on mass-balance calculations and Klason lignin analysis, the cell wall incorporation of coniferyl alcohol into

Table 1 Concentrations of lignin, *p*-coumarate (*p*CA), ferulate (FA), diferulate (DFA), and total ferulates (TFA) in maize cell walls artificially lignified with coniferyl alcohol and with sinapyl alcohol (SA) under varying levels of cell wall feruloylation and methyl *p*-coumarate (Me-pCA) addition

AIP[a]	Precursors[b]		Klason lignin[c]	Ester-linked[d]				Cross-linked[e]
	Monolignol	Me-pCA		*p*-CA	FA	DFA	TFA	TFA
Cell walls lignified with coniferyl alcohol								
0	0.57	0	267.2	0.08	0.45	0.42	0.87	14.54
Cell walls lignified with sinapyl alcohol								
0	0.57	0	86.0	0.09	0.83	0.92	1.75	13.66
0	0.57	0.028	92.6	0.32	0.88	0.87	1.74	13.67
5	0.57	0	57.2	0.07	0.17	0.47	0.64	2.57
5	0.57	0.028	61.9	0.22	0.14	0.42	0.57	2.64
Analysis of variance for cell walls lignified with sinapyl alcohol								
AIP			**	**	**	**	**	**
Precursors			*	**	NS	NS	NS	NS
AIP × precursors			NS	**	NS	NS	NS	NS
CV (%)			1.5	4.4	10.3	10.5	10.2	1.5

NS not significant

*, ** significant at the 0.05, and 0.01 levels of probability, respectively

[a] Concentration (μg/ml) of 2-aminoindan-2-phosphonic acid (AIP) added to cell suspensions of maize to limit feruloylation of cell walls

[b] Precursors (mmol) added to lignify ~280 mg of cell walls

[c] Concentration of acid-insoluble Klason lignin (mg/g cell wall)

[d] Ester-linked hydroxycinnamates (mg/g cell wall) released by alkaline hydrolysis

[e] TFA cross-linked to lignin (mg/g cell wall) estimated as the difference in alkali-labile acids recovered from nonlignified and artificially lignified cell walls

guaiacyl lignin was greater than that of sinapyl alcohol into syringyl lignin (Tables 2, 3). Adding sinapyl *p*-coumarate depressed the proportion of precursors incorporated into cell walls but only syringyl lignin concentrations were reduced. The apparent incorporation of sinapyl *p*-coumarate into cell walls, based on the recovery of alkali-labile *p*-coumarate, was lower than the overall incorporation of precursors and less for syringyl than for guaiacyl lignins. Dehydrogenation products recovered from reaction media and filtrate following lignification were also enriched in *p*-coumarates (^{1}H-NMR data not shown), further indicating that sinapyl *p*-coumarate was poorly incorporated into cell walls. Lignin concentrations were the same at both levels of precursor addition, but average incorporation declined from 80 to 59% and the apparent incorporation of sinapyl *p*-coumarate declined from 43 to 31% as precursor levels increased (data not shown). Alkaline hydrolysis of cell walls and filtrate recovered about 80% of the *p*-coumarate added as sinapyl *p*-coumarate (Table 2). Based on guaiacol staining, peroxidase activity was not limiting during lignification with monolignols but the inclusion of sinapyl *p*-coumarate unexpectedly accelerated peroxidase inactivation.

Prior to artificial lignification, primary maize cell walls contained 0.37 mg/g of *p*-coumarate, 15.2 mg/g of ferulate, and 5.7 mg/g of diferulates ester-linked to xylans. Lignification with monolignols reduced concentrations of *p*-coumarate by 40% and ferulate + diferulates by ~90%, suggesting that *p*-hydroxycinnamates esterified to xylans became oxidatively coupled to lignin by alkali-resistant bonds (data not shown). Adding sinapyl *p*-coumarate increased alkali-labile *p*-coumarate concentrations, particularly in cell walls lignified with coniferyl alcohol (Table 3). Conversely, when Klason lignin was used as a covariate, adjusted *p*-coumarate concentrations were similar for cell walls lignified with coniferyl alcohol or with sinapyl alcohol (data not shown). Concentrations of alkali-labile ferulate were greater in cell walls lignified with sinapyl alcohol than with coniferyl alcohol, while the converse was true for diferulates (Table 3). Adding sinapyl *p*-coumarate dramatically increased alkali-labile ferulate and diferulate concentrations and depressed cross-linking, particularly in cell walls lignified with sinapyl alcohol; using Klason lignin as a covariate did not alter these results (data not shown). Similar quantities of alkali-labile *p*-coumarate, ferulate, and diferulates, were recovered from cell walls lignified with both levels of precursor addition (data not shown).

Lignification with varying levels of ferulate and sinapyl *p*-coumarate

In the third study, primary cell walls from maize cell suspensions with normal or reduced feruloylation were

Table 2 Cell wall peroxidase activity and recovery of precursors and *p*-coumarate in maize cell walls (CW) artificially lignified with monolignols and sinapyl *p*-coumarate (SA-pCA)

Precursors[a]		Peroxidase[b]	Recovery				
			Precursors[c]		*p*-Coumarate[d]		
Monolignol	SA-*p*CA		Added	CW	Added	CW	Filtrate
Sinapyl alcohol							
0.75	0	Definite	158	70.6			
0.50	0.25	None	195	41.8	38.1	30.3	48.6
Coniferyl alcohol							
0.75	0	Weak	135	85.7			
0.50	0.25	None	180	66.8	39.1	43.8	31.6
Analysis of variance							
Monolignol				**		*	†
SA-*p*CA				**			
Monolignol × SA-*p*CA				NS			
CV (%)				5.7		1.6	4.7

NS not significant

*, **, † are significant at the 0.05, 0.01, and 0.1, levels of probability, respectively

[a] Average quantity of precursors (mmol) added to lignify ~0.9 g of cell walls. Data are averaged over two rates (0.6 and 0.9 mmol) of precursor addition

[b] Reaction of cell wall peroxidase with guaiacol and H_2O_2 at the end of precursor addition

[c] Quantity of precursors added (mg) to medium and the proportion (%) polymerized into cell walls as estimated by the difference in mass between nonlignified and artificially lignified walls

[d] Quantity of *p*-coumarate added (mg) to the reaction medium and the proportion (%) recovered in cell walls and in combined filtrates of reaction medium and cell wall washes as determined by alkaline hydrolysis

Table 3 Concentrations of lignin, *p*-coumarate (*p*CA), ferulate (FA), diferulate (DFA), and total ferulates (TFA) in maize cell walls artificially lignified with monolignols and sinapyl *p*-coumarate (SA-pCA)

Precursors[a]		Lignin[b]			Alkali-labile[c]				Cross-linked[d]
Monolignol	SA-pCA	Predicted	*Δ*Mass	Klason	*p*CA	FA	DFA	TFA	TFA
Sinapyl alcohol									
0.75	0	151.0	108.5	113.8	0.19	0.80	1.36	2.13	18.75
0.50	0.25	179.8	81.2	90.4	11.74	4.47	4.20	8.67	12.20
Coniferyl alcohol									
0.75	0	132.0	112.1	140.8	0.25	0.58	2.12	2.70	18.20
0.50	0.25	168.4	116.2	144.5	16.88	2.24	3.09	5.33	15.55
Analysis of variance[e]									
Monolignol			**	**	*	**	NS	**	**
SA-pCA			**	NS	**	**	**	**	**
Monolignol × SA-pCA			**	†	*	**	**	**	**
CV (%)			2.9	5.7	14.3	9.3	7.8	5.3	1.6

NS not significant

*, **, † are significant at the 0.05, 0.01, and 0.1 levels of probability, respectively

[a] Average quantity of precursors (mmol) added to lignify ~0.9 g of cell walls. Data are averaged over two rates (0.6 and 0.9 mmol) of precursor addition

[b] Lignin content (mg/g cell wall) predicted for complete polymerization of precursors into cell walls, and as estimated by the *Δ* cell wall mass and the Klason procedure

[c] Ester-linked hydroxycinnamates (mg/g cell wall) released by alkaline hydrolysis

[d] TFA cross-linked to lignin (mg/g cell wall) estimated as the difference in alkali-labile acids recovered from nonlignified and artificially lignified cell walls

lignified with varying levels sinapyl *p*-coumarate to further investigate how ferulate and *p*-coumarate esters interact during lignification. Prior to lignification, H_2O_2 was added to fully dimerize ferulates via wall-bound peroxidase, thereby permitting the incorporation of ferulate monomers and dimers into lignin to be tracked. Following H_2O_2 treatment, cell walls from normal cell suspensions contained 0.51 mg/g of *p*-coumarate, 9.16 mg/g of ferulate, and 3.16 mg/g of diferulates. Growing cell suspensions with the highest quantity of AIP (10 μg/ml) decreased alkali-labile cell wall *p*-coumarates by 64%, ferulate monomers by 88%, and diferulates by 73% (data not shown). Precursors were added to form mixed guaiacyl-syringyl lignins at 0.75 and 1.5-fold the level found in mature maize parenchyma (Morrison et al. 1998). Sinapyl *p*-coumarate was added at 0, 0.5, and 1.0 times its presumed abundance in maize lignin (Ralph et al. 1994).

In general, the impact of sinapyl *p*-coumarate addition on precursor incorporation, peroxidase activity, and recovery of alkali-labile *p*-coumarate from lignified cell walls were comparable to the previous experiment (data not shown). Reductions in cell wall feruloylation did not affect the overall incorporation of precursors into cell walls (which averaged 55%), but the recovery alkali-labile *p*-coumarate derived from sinapyl *p*-coumarate declined from 43 to 35%.

When averaged over the two levels of precursor addition, Klason and mass-balance lignin concentrations were similar among treatments (Table 4). However, with lower precursor additions, Klason lignin concentrations increased from 100 to 113 mg/g when sinapyl *p*-coumarate was added with monolignols (data not shown). Since the overall efficiency of precursor incorporation was similar among treatments, lignin concentrations increased because sinapyl *p*-coumarate has a greater mass than sinapyl alcohol. At higher precursor levels, sinapyl *p*-coumarate depleted peroxidase activity and this decreased Klason lignin concentrations from 147 to 131 mg/g. Similar trends ($P < 0.10$) were observed for lignin concentrations as estimated by mass balance calculations.

As expected, adding sinapyl *p*-coumarate with monolignols increased alkali-labile *p*-coumarate concentrations in cell walls (Table 4). As highlighted by a significant AIP × precursor interaction, cell walls with lower feruloylation contained slightly less alkali-labile *p*-coumarate derived from sinapyl *p*-coumarate. As noted in the previous experiment, adding sinapyl *p*-coumarate with monolignols increased alkali-labile ferulate and diferulate concentrations in cell walls, indicating a reduced cross-linking of xylans to lignin via ferulate and diferulate. Sinapyl *p*-coumarate interfered with the incorporation of diferulates to a greater degree than ferulate with 8-5-coupled diferulate

Table 4 Influence of cell wall feruloylation and sinapyl *p*-coumarate (SA-*p*CA) on the concentrations of lignin, *p*-coumarate (*p*CA), ferulate (FA), diferulate (DFA), and total ferulates (TFA) in maize cell walls artificially lignified with coniferyl alcohol (CA) and sinapyl alcohol (SA)

AIP[a]	Precursors[b]			Lignin[c]			Ester-linked[d]				Cross-linked[e]		
	CA	SA	SA-*p*CA	Predicted	Δ mass	Klason	*p*CA	FA	DFA	TFA	FA	DFA	TFA
0	0.45	0.60	0	117.7	74.7	124.2	0.26c	0.28c	0.62c	0.90c	8.13a	3.43a	11.55a
0	0.45	0.45	0.15	128.7	72.3	126.4	5.73b	0.45b	1.06b	1.50b	7.97b	2.99b	10.96b
0	0.45	0.30	0.30	139.4	63.2	121.7	11.74a	0.66a	1.48a	2.14a	7.75c	2.57c	10.32c
2	0.45	0.60	0	123.7	83.4	123.0	0.12c	0.10b	0.26c	0.36c	3.84a	2.37a	6.21a
2	0.45	0.45	0.15	135.0	72.9	116.4	5.03b	0.16a	0.49b	0.65b	3.77b	2.14b	5.91b
2	0.45	0.30	0.30	146.0	78.1	121.1	11.28a	0.21a	0.70a	0.92a	3.72b	1.92c	5.65c
10	0.45	0.60	0	122.2	76.1	123.2	0.12c	0.04a	0.06b	0.10b	0.72a	0.98a	1.70a
10	0.45	0.45	0.15	133.5	71.1	121.0	4.22b	0.04a	0.15ab	0.19ab	0.72a	0.89ab	1.61ab
10	0.45	0.30	0.30	144.5	83.4	124.8	10.04a	0.05a	0.24a	0.29a	0.71a	0.79b	1.51b
Analysis of variance													
AIP					NS	NS	*	*	†	*	**	†	**
Precursors					NS	NS	**	**	**	**	**	**	**
AIP × precursors					NS	NS	*	**	†	**	**	**	**
CV (%)					9.8	3.3	5.0	9.8	9.7	8.0	0.5	2.7	1.0

Means within a column and AIP level, followed by a different letter are significantly different according to Fisher's protected LSD ($P = 0.05$)

NS not significant

†, *, ** are significant at the 0.1, 0.05, and 0.01 levels of probability, respectively

[a] Concentration (μg/ml) of 2-aminoindan-2-phosphonic acid (AIP) added to cell suspensions of maize to limit feruloylation of cell walls

[b] Average quantity of precursors (mmol) added to lignify 1.5 g of cell walls. Data are averaged over two rates (0.7 and 1.4 mmol) of precursor addition

[c] Lignin content (mg/g cell wall) predicted with complete polymerization of precursors into cell walls and estimated by the Δ cell wall mass and by the Klason procedure

[d] Ester-linked hydroxycinnamates (mg/g cell wall) released by alkaline hydrolysis

[e] Hydroxycinnamates cross-linked to lignin (mg/g cell wall) estimated as the difference in alkali-labile acids recovered from nonlignified and artificially lignified cell walls

being most affected. A significant AIP × precursor interaction suggests that the adverse affects of sinapyl *p*-coumarate on ferulate and diferulate incorporation diminished as feruloylation of cell walls decreased. The impact of sinapyl *p*-coumarate on ferulate and diferulate incorporation was also less pronounced than in the previous study where cell walls had higher inherent levels of feruloylation.

p-Coumarate coupling in preformed lignins

A fourth study was conducted to determine if *p*-coumarate esters in naturally formed maize lignin can participate in oxidative coupling reactions. Monolignols were added to isolated pith cell walls from partially or fully elongated internodes to potentially increase in situ lignin concentrations from an initial average of 100 mg/g to over 300 mg/g. Based on changes in cell wall mass, monolignol incorporation into cell walls averaged 75% for coniferyl alcohol and 20% for sinapyl alcohol. Oxidative coupling reactions reduced alkali-labile ferulate + diferulate concentrations, particularly when pith cell walls were lignified with coniferyl alcohol (Table 5). While ferulate xylan esters in pith cell walls readily underwent oxidative coupling reactions, *p*-coumarates esterified to pith lignin did not, as evidenced by their unchanged recovery by alkaline hydrolysis following various oxidative treatments. The recovery of alkali-labile *p*-coumarate was also unaffected when an isolated maize lignin was suspended in buffer or dissolved in dioxane:buffer and subjected to a similar series of oxidative coupling treatments (Table 5).

Discussion

The in vitro polymerization of monolignols into primary walls by in situ peroxidases has proved to be a good model particularly for primary cell wall lignification in grasses (Grabber 2005). In our studies, the polymerization of sinapyl alcohol into wall-bound lignins was one-third that of coniferyl alcohol when each monolignol was added in

Table 5 Concentrations of lignin and alkali-labile *p*-hydroxycinnamates in maize pith cell walls and isolated maize lignin after H_2O_2 treatment or artificial lignification with H_2O_2 and sinapyl alcohol (SA) or coniferyl alcohol (CA)

Treatment	Maize pith walls[a]			Maize lignin *p*-coumarate[b]
	Klason lignin	Ferulate and diferulates	*p*-Coumarate	
Untreated control	99.1a[c]	13.5a	19.8a	156.0a
H_2O_2	101.8a	11.8b	19.7a	155.2a
H_2O_2 + SA	118.0b	10.2c	19.8a	147.2a
H_2O_2 + CA	249.6c	4.6d	18.2a	148.6a
CV (%)	2.8	7.5	5.4	9.7

[a] Concentrations (mg/g cell wall) of ferulate, diferulate, and *p*-coumarate were adjusted to account for changes in mass due to polymerization of monolignols into cell walls

[b] Concentrations (mg/g lignin) are averaged over reactions carried out in buffer (pH 5.5) or buffer:dioxane (1:1). Solvent effects on *p*-coumarate concentrations were not significant ($P > 0.05$)

[c] Mean values within a column followed by a different letter are significantly different according to Fisher's protected LSD ($P = 0.05$)

excess to nonlignified cell walls isolated from maize cell suspensions (Table 1). Similar results were obtained with partially lignified primary-walled pith cell walls prepared from partially to fully elongated internodes from maize (Table 5). The results with pith are particularly noteworthy since the high *p*-coumarate content of the untreated controls suggests syringyl units were actively being incorporated into lignin (Morrison et al. 1998), prior to our isolation of pith for lignification experiments. In the current study and in previous work (Funk et al. 2006), the maximum amount of syringyl-rich lignins formed in artificially lignified walls (~140 mg/g) corresponds closely to the maximal lignin content observed for maize pith (Morrison et al. 1998). Therefore, primary walls of maize have a low capacity to form syringyl-rich lignins, apparently because sinapyl alcohol is a poor substrate for maize peroxidases (Hatfield et al. 1997). Incidentally, although syringyl lignins are more acid-soluble than guaiacyl lignins (Musha and Goring 1974), estimates of both types of lignin by the Klason method were quite comparable to that predicted by mass-balance calculations (Table 3).

Catalytic amounts of *p*-hydroxycinnamic acids or their simple esters (e.g., ethyl ferulate and methyl *p*-coumarate) are reported to enhance sinapyl alcohol oxidation by apoplastic peroxidases in maize and other species (Takahama et al. 1996; Hatfield et al. 1997). When primary maize walls were lignified with an excess of sinapyl alcohol (2 mmol/g), we found that an 80% reduction in naturally esterified cell wall ferulates (0.056 mmol/g) reduced syringyl lignin formation by 33% (Table 1). One-third of this decline was due to fewer ferulates being incorporated into lignin. Regardless of the degree of feruloylation, adding catalytic amounts of methyl *p*-coumarate (0.1 mmol/g cell wall) with sinapyl alcohol increased syringyl lignin formation in cell walls by only 8%. Thus, at comparable levels, soluble *p*-coumarate esters are less effective than wall-bound ferulate esters for stimulating syringyl lignin polymerization into maize cell walls. The overall impact of both esters on syringyl lignin formation was, however, very modest.

In most of our cell wall model experiments, adding physiological levels of sinapyl *p*-coumarate [the likely precursor of *p*-coumarates on lignin (Ralph et al. 1994; Grabber et al. 1996a; Morrison et al. 1998)] with monolignols adversely affected precursor polymerization into cell walls (Tables 2, 3, 4). As noted previously (Ferrer and Barcelo 1994), peroxidase activity declined during the course of cell wall lignification. Surprisingly, we found that adding sinapyl *p*-coumarate with monolignols accelerated peroxidase inactivation (as detected by guaiacol staining) and this depressed lignification, To our knowledge, this antagonistic effect has not been previously reported but it might in part be due to partial hydrolysis of sinapyl *p*-coumarate and reaction of free *p*-coumarate with the prosthetic heme in peroxidase (Huang et al. 2004). Yet even when adequate peroxidase activity was present, sinapyl *p*-coumarate had no apparent effect on cell wall lignification. Reductions in cell wall feruloylation adversely affected sinapyl *p*-coumarate incorporation, further undermining the proposed role of *p*-coumarates for enhancing syringyl lignin formation in cell walls. Thus, while ferulates can act as initiation sites for lignification (Ralph et al. 1995; Grabber et al. 2002) and both *p*-coumarate and ferulate esters enhance sinapyl alcohol oxidation (Takahama et al. 1996; Hatfield et al. 1997), they do not—at least under the conditions employed here—have much impact on syringyl-lignin formation in cell walls. Thus unknown factors, absent from primary cell walls (or at least absent from our primary wall model), promote syringyl lignin formation, particularly as observed in secondary maize cell walls (Chesson et al. 1997).

Following artificial lignification with monolignols and sinapyl *p*-coumarate, Klason lignin, and alkali-labile *p*-coumarate concentrations in primary maize walls (Tables 3,4) were comparable to that observed in mature maize pith (Morrison et al. 1998). In our model studies, 31–44% of the *p*-coumarate added to the reaction medium as sinapyl *p*-coumarate was recovered by alkaline hydrolysis of lignified cell walls. Although alkaline hydrolysis of filtrates collected after lignification yielded additional *p*-coumarate, some was not recovered presumably due to oxidative coupling to lignin by alkali-stable ether-linked or carbon-carbon bonds. High-temperature alkaline hydrolysis of grass cell walls has also indicated that some *p*-coumarates are ether-linked to lignin (Lam et al. 1996; Morrison et al. 1998), but most are probably terminal units attached to lignin by only ester-linkages (Ralph et al. 1994). Previously, we speculated that *p*-coumarates occur mainly as terminal units in syringyl-rich maize lignin because differing oxidation potentials of *p*-coumarate and sinapyl alcohol should prevent their cross-coupling (Ralph et al. 1994). But in our current study, copolymerization of sinapyl *p*-coumarate with coniferyl or sinapyl alcohols yielded comparable recoveries of *p*-coumarate following alkaline hydrolysis, suggesting that *p*-coumarate had a similar propensity to cross-couple with syringyl and guaiacyl units in lignin. Thus, other factors probably control the coupling reactions of *p*-coumarate during lignification.

In studies with maize pith walls containing *p*-coumaroylated lignins, *p*-coumarates did not undergo oxidative coupling reactions with each other or with added monolignols (Table 5). Oxidative coupling reactions did occur in pith cell walls, as demonstrated by the reduced recovery of alkali-labile ferulate and diferulates and the marked increase in wall-bound lignins formed especially with added coniferyl alcohol. Carrying out similar reactions with a *p*-coumaroylated lignin isolated from maize internodes yielded similar results. In this case, we carried out reactions with lignin suspended in buffer or dissolved in dioxane–buffer to improve the accessibility of free phenolic groups to oxidative reactions. Surprisingly, even with dissolved lignin, *p*-coumarate units still did not undergo oxidative coupling. Thus, once incorporated by their syringyl moiety into lignin, pendant *p*-coumarate units are completely disinclined to undergo oxidative coupling reactions. This would seemingly preclude *p*-coumarate units on lignin in having a role in oxidative cross-linking of cell walls or in lignin polymerization in response to plant pathogen attack or insect herbivory.

In our model studies, copolymerization of sinapyl *p*-coumarate with monolignols unexpectedly reduced ferulate and diferulate incorporation into lignin, especially for lignins rich in syringyl units (Tables 3, 4). We also observed that naturally lignified maize pith had relatively high concentrations of alkali-labile ferulates and diferulates, much of which became nonrecoverable upon the addition of hydrogen peroxide and monolignols (Table 5). Thus, much of the ferulate and diferulates in maize pith were capable of undergoing oxidative coupling but did not during the formation of *p*-coumaroylated lignin. Because ferulates normally copolymerize quite readily with monolignols, we previously speculated that alkali-labile ferulate concentrations might be inversely related to the distribution of lignin in cell walls (Grabber et al. 2004). Continuous deposition of feruloylated xylans during cell wall synthesis (MacAdam and Grabber 2002; Jung 2003) may also account for some of the alkali-labile ferulates and diferulates in lignified cell walls. However, the results of our current study strongly indicate that sinapyl *p*-coumarate directly interferes with oxidative coupling reactions of ferulate and diferulates during lignification. The antagonistic effect of sinapyl *p*-coumarate on ferulate copolymerization into wall-bound lignins has not, to our knowledge, been previously reported, but it may in part explain why significant quantities of alkali-labile ferulate can be recovered even from highly lignified grass tissues (Grabber et al. 1991; Morrison et al. 1998; Hatfield et al. 1999).

In ongoing work, we are investigating how temporal aspects of guaiacyl and syringyl lignin formation (Terashima et al. 1993) influence the incorporation of sinapyl *p*-coumarate into cell walls and the type of coupling structures it forms. In addition, we will assess whether sinapyl *p*-coumarate contributes to the relatively low molecular weight and high extractability of grass lignins and evaluate its impact cell wall degradability by ruminal microflora. We are conducting these studies under conditions that minimize dioxane additions, sinapyl *p*-coumarate hydrolysis, and peroxidase inactivation during lignification—factors somewhat limiting our modeling of *p*-coumaroylated lignin formation in studies described herein. We hope these studies will provide some insight into the functional role of extensive lignin acylation by *p*-coumarate in grasses.

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References

Bergvinson DJ, Arnason JT, Hamilton RI (1997) Phytochemical changes during recurrent selection for resistance to the European corn borer. Crop Sci 37:1567–1572

Bily AC, Reid LM, Taylor JH, Johnston D, Malouin C, Burt AJ, Bakan B, Regnault-Roger C, Pauls KP, Arnason JT, Philogene BJR

(2003) Dehydrodimers of ferulic acid in maize grain pericarp and aleurone: resistance factors to *Fusarium graminearum*. Phytopathology 93:712–719

Bunzel M, Ralph J, Marita JM, Hatfield RD, Steinhart H (2001) Diferulates as structural components in soluble and insoluble cereal dietary fibre. J Sci Food Agric 81:653–660

Casler MD, Jung HJG (1999) Selection and evaluation of smooth bromegrass clones with divergent lignin or etherified ferulic acid concentration. Crop Sci 39:1866–1873

Chesson A, Provan GJ, Russell W, Scobbie L, Chabbert B, Monties B (1997) Characterisation of lignin from parenchyma and sclerenchyma cell walls of the maize internode. J Sci Food Ag 73:10–16

Ferguson LR, Lim IF, Pearson AE, Ralph J, Harris PJ (2003) Bacterial antimutagenesis by hydroxycinnamic acids from plant cell walls. Mutation Research 542:49–58

Ferrer MA, Barcelo AR (1994) Inactivation of cell wall acidic peroxidase isoenzymes during the oxidation of coniferyl alcohol in *Lupinus*. Phytochemistry 36:1161–1163

Funk C, Weber P, Thilker J, Grabber JH, Steinhart H, Bunzel M (2006) Influence of lignification and feruloylation of maize cell walls on the adsorption of heterocyclic aromatic amines. J Ag Food Chem 54:1860–1867

Garcia-Conesa MT, Wilson PD, Plumb GW, Ralph J, Williamson G (1999) Antioxidant properties of 4,4′-dihydroxy-3,3′-dimethoxy-β,β′-bicinnamic acid (8-8-diferulate non-cyclic form). J Sci Food Agric 79:379–384

Gordon AH, Lomax JA, Dalgarno K, Chesson A (1985) Preparation and composition of mesophyll, epidermis and fibre cell walls from perennial ryegrass (*Lolium perenne*) and Italian ryegrass (*Lolium multiflorum*). J Sci Food Agric 36:509–519

Grabber JH (2005) How do lignin composition, structure, and cross-linking affect degradability? A review of cell wall model studies. Crop Sci 45:820–831

Grabber JH, Jung GA, Hill RR (1991) Chemical composition of parenchyma and sclerenchyma cell walls isolated from orchardgrass and switchgrass. Crop Sci 31:1058–1065

Grabber JH, Quideau S, Ralph J (1996a) *p*-Coumaroylated syringyl units in maize lignin; implications for β-ether cleavage by thioacidolysis. Phytochemistry 43:1189–1194

Grabber JH, Ralph J, Hatfield RD, Quideau S, Kuster T, Pell AN (1996b) Dehydrogenation polymer-cell wall complexes as a model for lignified grass walls. J Agric Food Chem 44:1453–1459

Grabber JH, Ralph J, Hatfield RD (1998) Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. J Agric Food Chem 46:2609–2614

Grabber JH, Ralph J, Hatfield RD (2000) Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. J Agric Food Chem 48:6106–6113

Grabber JH, Ralph J, Hatfield RD (2002) Model studies of ferulate-coniferyl alcohol cross-product formation in primary maize walls: implications for lignification in grasses. J Agric Food Chem 50:6008–6016

Grabber JH, Ralph J, Lapierre C, Barrière Y (2004) Genetic and molecular basis of grass cell wall degradability. I. Lignin-cell wall matrix interactions C R Biologies 327:455–465

Hatfield RD, Jung HG, Ralph J, Buxton DR, Weimer PJ (1994) A comparison of the insoluble residues produced by the Klason lignin and acid detergent lignin procedures. J. Sci. Food Agric. 65:51–58

Hatfield RD, Grabber J, Ralph J (1997) A potential role of sinapyl *p*-coumarate in grass lignin formation. Annual meeting of the American society of plant physiologists, Vancouver, p 86

Hatfield RD, Wilson JR, Mertens DR (1999) Composition of cell walls isolated from cell types of grain sorghum stems. J Sci Food Agric 79:891–899

Huang L, Colas C, Ortiz de Montellano PR (2004) Oxidation of carboxylic acids by horseradish peroxidase results in prosthetic heme modification and inactiviation. J Am Chem Soc 126:12865–12873

Jung HJG (2003) Maize stem tissues: ferulate deposition in developing internode cell walls. Phytochemistry 63:543–549

Lam TBT, Iiyama K, Stone B (1996) Lignin and hydroxycinnamic acids in walls of brown midrib mutants of sorghum, pearl millet and maize stems. J Sci Food Agric 71:174–178

Lu F, Ralph J (1998a) Facile synthesis of 4-hydroxycinnamyl *p*-coumarates. J Agric Food Chem 46:2911–2913

Lu F, Ralph J (1998b) Highly selective syntheses of coniferyl and sinapyl alcohols. J Agric Food Chem 46:1794–1796

Lu F, Ralph J (2005) Novel β–β-structures in natural lignins incorporating acylated monolignols. In: 13th international symposium on wood, fiber, and pulping chemistry. APPITA, Auckland, p 233–237

MacAdam JW, Grabber JH (2002) Relationship of growth cessation with the formation of diferulate cross-links and *p*-coumaroylated lignins in tall fescue. Planta 215:785–793

Migne C, Prensier G, Utille JP, Angibeaud P, Cornu A, Grenet E (1998) Immunocytochemical localisation of para-coumaric acid and feruloyl-arabinose in the cell walls of maize stem. J Sci Food Agric 78:373–381

Morrison TA, Jung HG, Buxton DR, Hatfield RD (1998) Cell-wall composition of maize internodes of varying maturity. Crop Sci 38:455–460

Musel G, Schindler T, Bergfeld R, Ruel K, Jacquet G, Lapierre C, Speth V, Schopfer P (1997) Structure and distribution of lignin in primary and secondary cell walls of maize coleoptiles analyzed by chemical and immunological probes. Planta 201:146–159

Musha Y, Goring DAI (1974) Klason and acid soluble lignin content of hardwoods. Wood Sci 7:133–134

Myton KE, Fry SC (1994) Intraprotoplasmic feruloylation of arabinoxylans in *Festuca arundinacea* cell cultures. Planta 193:326–330

Ralph J, Hatfield RD, Quideau S, Helm RF, Grabber JH, Jung H-JG (1994) Pathway of *p*-coumaric acid incorporation into maize lignin as revealed by NMR. J Am Chem Soc 116:9448–9456

Ralph J, Grabber JH, Hatfield RD (1995) Lignin-ferulate cross-links in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. Carbohydr Res 275:167–178

Santiago R, Malvar RA, Baamonde MD, Revilla P, Souto XC (2005) Free phenols in maize pith and their relationship with resistence to *Sesamia nonagrioides* (Lepidoptera: Noctuidae) attack. J Econ Entomol 98:1349–1356

Santiago R, Butron A, Arnason JT, Reid LM, Souto XC, Malvar RA (2006) Putative role of pith cell wall phenylpropanoids in *Sesamia nonagrioides* (Lepidoptera: Noctuidae) resistance. J Ag Food Chem 54:2274–2279

Saulnier L, Crepeau MJ, Lahaye M, Thibault JF, Garcia-Conesa MT, Kroon PA, Williamson G (1999) Isolation and structural determination of two 5,5′-diferuloyl oligosaccharides indicate that maize heteroxylans are covalently cross-linked by oxidatively coupled ferulates. Carbohydr Res 320:82–92

Schooneveld-Bergmans MEF, Dignum MJW, Grabber JH, Beldman G, Voragen AGJ (1999) Studies on the oxidative cross-linking of feruloylated arabinoxylans from wheat flour and wheat bran. Carbohydr Polymers 38:309–317

Schopfer P (1996) Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles. Planta 199:43–49

Steel RGD, Torrie JH (1980) Principles and procedures of statistics. McGraw-Hill Publishing Co., New York, pp 633

Sukalovic VHT, Vuletic M, Vucinic Z (2005) The role of *p*-coumaric acid in oxidative and peroxidative cycle of the ionically bound peroxidase of the maize root cell wall. Plant Sci 168:931–938

Takahama U, Oniki T, Shimokawa H (1996) A possible mechanism for the oxidation of sinapyl alcohol by peroxidase-dependent reactions in the apoplast: enhancement of the oxidation by hydroxycinnamic acids and components of the apoplast. Plant Cell Physiol 37:499–504

Terashima N, Fukushima K, He L-F, Takabe K (1993) Comprehensive model of the lignified plant cell wall. In: Jung HG, Buxton DR, Hatfield RD, Ralph J (eds) Forage cell wall structure and digestibility. ASA-CSSA-SSSA, Madison, pp 247–270